

Inhibition of apoptosis in rat mesangial cells by tissue inhibitor of metalloproteinase-1

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Inhibition of apoptosis in rat mesangial cells by tissue inhibitor of metalloproteinase-1.

Background. Tissue inhibitor of metalloproteinase-1 (TIMP-1) is an important inhibitor of extracellular matrix degradation. Recently, it was reported that TIMP-1 also could inhibit apoptosis in B type lymphocyte. This study was designed to examine the effects of TIMP-1 on mesangial cell apoptosis.

Methods. The full-length cDNA of TIMP-1 was cloned and used to construct two recombinant vectors, TIMP-1S and TIMP-1AS, encoding sense TIMP-1 and antisense TIMP-1, respectively. The vectors were transfected into rat mesangial cells (RMC) and their expressions detected by Northern and Western blotting. Apoptosis was induced by serum deprivation, and was monitored for DNA fragmentation by TUNEL assay and DNA laddering. In addition, the expression of endogenous TIMP-1, matrix metalloprotein-2 (MMP-2), and MMP-9, as well as apoptosis-related genes *Bcl-2* and *Bax* were investigated.

Results. TIMP-1AS transfection induced a suppression of TIMP-1 expression accompanied by an earlier onset of apoptosis, and TIMP-1S transfection induced TIMP-1 over-expression accompanied by a much later onset of apoptosis. A neutralizing antibody of TIMP-1 restored the sensitivity of TIMP-1S-transfected RMC to serum deprivation, but a synthetic matrix metalloproteinase inhibitor BB-94 did not influence the sensitivity of TIMP-1S-transfected RMC to serum deprivation. Finally, TIMP-1 over-expression inhibited the expression of *Bax* but with no effect on the expression of *Bcl-2*.

Conclusion. TIMP-1 inhibits the serum deprivation-induced apoptosis in RMC, in which *Bax* might be involved.

Glomerulosclerosis, a process that leads to chronic renal failure in a variety of human kidney disorders, has been attributed to cell proliferation and extracellular matrix (ECM) accumulation in the glomeruli [1–4]. Cell proliferation is the net result of the imbalance between cell proliferation and apoptosis [5], while ECM accumu-

lation the net result of the imbalance between ECM synthesis and degradation [6–8].

Apoptosis and proliferation are mechanisms regulating the homeostasis of cell number [5]. It has been well accepted that over-proliferation of glomerular mesangial cells can increase the accumulation of ECM promoting progression of glomerulosclerosis [2]. It is now known that glomerular mesangial cell apoptosis is the major mechanism for resolution of glomerular hypercellularity in experimental glomerulonephritis [5, 9].

Apart from apoptosis, ECM degradation is another potential way for prevention of glomerulosclerosis. Components of ECM can be degraded by matrix metalloproteinases (MMPs), a group of structurally related zinc metallo-enzymes [6]. MMP activities are negatively regulated by tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site of MMPs [10]. The balance between MMPs and TIMPs determines the net activity of MMPs/TIMPs system [11]. Among the four TIMP genes reported to date, *TIMP-1* was the first identified and has been generally accepted as a key factor in ECM accumulation through its MMP inhibiting activity [12].

Interestingly, besides its MMP-inhibiting activity, *TIMP-1* also has some MMP-independent effects, such as erythroid potentiating activity and cell growth promoting activity in a wide range of cells [13, 14]. More recently, another important novel function of *TIMP-1* was discovered: it suppresses apoptosis in B cells [15]. It is of interest to know the potential role of *TIMP-1* in the apoptosis of renal cells, as the balance between mesangial cell proliferation and apoptosis is a very important event in many renal diseases and has been associated with the subsequent development of glomerulosclerosis [16]. In this study, our aim was to determine whether *TIMP-1* contributes to apoptosis in rat mesangial cells (RMC). We developed two transfected RMC models that had either increased or decreased TIMP-1 expression to investigate the role of TIMP-1 in the apoptosis of RMC induced by serum deprivation.

Key words: transfection, apoptosis, *Bcl-2*, *Bax*, cell death, extracellular matrix, glomerulosclerosis.

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METHODS

Materials and reagents

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and were of the highest grade. TRIzol reagent was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Restriction endonucleases and T₄ ligase were purchased from Promega Ltd (Madison, WI, USA). The liposomal transfection kit, DNA labeling kit, and apoptosis detection kit were from Boehringer Mannheim GMBH (Mannheim, Germany). The Superscript RNase H⁻Reverse Transcriptase kit was from Gibco BRL, Life Technologies (Paisley, Scotland, UK); the DNA purification kit was purchased from Qiagen Ltd., (Hilden, Germany). The pcDNA3.1 vector, TOPO10 competent *E. coli* cells, and pCR[®]2.1 vector were purchased from Invitrogen (Groningen, The Netherlands). Synthetic MMP inhibitor BB-94 was from British Biotech (Oxford, UK). The TIMP-1 antibody was from Oncogene Research Products (Cambridge, MA, USA).

Primers were based on the published sequences. The following sequences were used. Human TIMP-1 sense, 5'-TTG AAT TCC CAC CAT GGC CCC CTT TGA GCC-3', antisense, 5'-GT GAATCC GCA GGA TTC AGG CTA TCT G-3' [17]. Rat TIMP-1 sense, 5'-TTT GCA TCT CTG GCC TCT-3', antisense, 5'-AAT GAC TGT CAC TCT CCA-5' [18]. Neo^r sense, 5'-CAA GAT GGA TTG CAC GCA GCA GG-3', antisense, 5'-CCC GCT CAG AAG AACTCG TC-3' [19]. Rat Bcl-2 sense, ACC GGG AGA TCG TGA TGA AGT-3', antisense, 5'-CGG TTC AGG TAC TCA GTC ATC C-3' [20]. Rat Bax sense, 5'-GAC ACC TGA GCT GAC CTT GG-3', antisense, 5'-GA GGA AGT CCA GTG TCC AGC-3' [21]. Rat MMP-2 sense, 5'-ATC TGG TGT CTC CCT TAC GG-3', antisense, 5'-GTG CAG TGA TGT CCG ACA AC-3' [22]. Rat MMP-9 sense, 5'-CAT GCG GCC GCC ATG AGT CC-3', antisense, 5'-TTG GAT CCA GTA TGT GAT GTT ATG ATG-3' [23].

Cloning of human sense TIMP-1 and antisense TIMP-1

The full-length human TIMP-1cDNA was isolated by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from normal human kidney tissues using TRIzol reagent following the protocol provided by Invitrogen Corp. First-strand cDNA was synthesized using an oligo dT primer (Gibco BRL), and PCR was carried out using Vent_R Taq polymerase (New England Biolabs, Beverly, MA, USA) at 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 60 seconds for the first 10 cycles, and then 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for two minutes for the secondary 15 cycles. The amplified DNA frag-

ment was cloned in the pCR[®]2.1 vector (Invitrogen). The plasmid was then purified and sequenced.

The sense and antisense TIMP-1 recombinant vectors were constructed through standard procedures described by Sambrook [24]. pCR[®]2.1-TIMP-1 was digested by EcoRI, and human TIMP-1 was inserted into the EcoRI site of a pcDNA3.1 expression vector. TIMP-1 orientation was confirmed by digestion with EcoRI and AvaI and the two expression vectors were obtained: TIMP-1S, encoding the full-length TIMP-1 sense, and TIMP-1AS, encoding the full-length TIMP-1 antisense.

Culture of RMC

Rat mesangial cells were cultured from the glomerular explants of adult Wistar rat kidneys using standard techniques [25]. The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in a 5% humidified CO₂ controlled atmosphere. Cultured cells were characterized by their typical stellate fusiform morphology, with positive staining for Thy1.1 antigen [26] and with resistance to the toxic effects of D-valine [27]. RMC at passage 3 was used in this study.

Transfection of TIMP-1S and TIMP-1AS

Rat mesangial cells were transfected with the empty pcDNA3.1 control vector, TIMP-1S-pcDNA3.1, or TIMP-1AS-pcDNA3.1 using DOTAP liposomal transfection reagent according to the Boehringer Mannheim protocol. Briefly, the passage 3 RMC was cultured in RPMI 1640 medium supplemented with 10% FBS, but with no antibiotics addition. RMC was sub-cultured 18 hours before transfection. The DOTAP/nucleic acid mixture (5 µg DNA and 30 µL DOTAP) was added in the culture medium and incubated for six hours. The antibiotic free medium was replaced with complete medium and the cells were incubated continuously. For stable transfection, the cells were subcultured 48 hours after transfection, and then 0.4 mg/mL geneticin was added, after which the cells were subsequently cultured in the presence of geneticin for 10 days. Monoclonal transfected cell lines were obtained by using a cloning cylinder and six cloned cell lines were established for the TIMP-1S transfected RMC (TIMP-1S/MC), seven cloned cell lines were established for TIMP-1AS (TIMP-1AS/MC), and three cloned cell lines were established for the vector transfected RMC (Vector/MC).

Genomic integration of human TIMP-1S and TIMP-1AS in RMCs

Genomic DNA was extracted from Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, or parental RMC. PCR for amplifying human TIMP-1 sense or TIMP-1 antisense was performed at 94°C for 30 seconds, 58°C for one minute,

and 72°C for one minute for 30 cycles. PCR for amplifying neo^r in pcDNA3 was performed at 94°C for 30 seconds, 61°C for 45 seconds, and 72°C for one minute, for 25 cycles.

RT-PCR and Northern blot analysis

Reverse transcription-PCR was performed to evaluate sense and antisense human TIMP-1 expression. Total RNA was extracted from the Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, or parental RMC. For detecting antisense human TIMP-1 expression only, the antisense primer of human TIMP-1 was used as reverse transcription primer in specific synthesis of the first stand of antisense hTIMP-1 cDNA. For detecting both sense and antisense hTIMP-1, an oligo dT primer was used as reverse transcription primer in synthesis of the first stand of hTIMP-1 cDNA. GAPDH primer was used as a control. RT-PCR was performed at 94°C for 30 seconds, 58°C for one minute, and 72°C for one minute, for 25 cycles.

Northern blotting was performed to determine the mRNA expressions of human TIMP-1S and AS, rat TIMP-1, rat MMP-2, rat MMP-9, rat Bcl-2 and rat Bax. A gel-electrophoresis was run with aliquots of RNA in 1% agarose-formaldehyde gels and later transferred to nylon membranes. These six cDNA probes were α -³²P randomly labeled for 24 hours and then hybridized. The Northern blots were exposed to an x-ray film at -70°C for a suitable time.

Western blot analysis for exogenous human TIMP-1 and endogenous rat TIMP-1 expression

Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, or parental RMC was washed with phosphate-buffered saline (PBS) for three times and incubated in lysis-buffer for 30 minutes. Supernatants were cleared of cell fragments by centrifugation and protein concentration determined by Coomassie bright blue assay. The sample protein was mixed with an equal volume of sample buffer, boiled for five minutes, electrophoresed on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel for 60 minutes at room temperature, and transferred to nitrocellulose filters (Amersham Pharmacia Biotech, Castle Hill, NSW, Australia). The membrane was blocked with TTBS solution containing 3% bovine serum albumin (BSA), incubated with mouse monoclonal antibodies against human TIMP-1 dilution of 1:200 at room temperature for 60 minutes and the binding was detected by the use of horseradish peroxidase (HRP)-conjugated secondary antibody system. Also, goat anti-rat polyclonal antibody was used to detect the endogenous rat TIMP-1 protein expression.

Apoptosis detection

The apoptosis of Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, or parental RMC was induced by serum deprivation. Briefly, 5×10^5 cells were rinsed three times in

PBS and then cultured in serum-free medium for 12 to 96 hours at 37°C. Apoptosis also was induced in these serum-deprived cells pre-treated with anti-TIMP-1 monoclonal antibody (5 µg/mL; Oncogene Research Products, Cambridge, MA, USA) or MMP inhibitor BB-94. Cells were monitored for apoptosis over extended periods of time (12 to 96 hours), and optimal time points for detection of apoptosis were determined.

Apoptosis was detected by DNA fragmentation using DNA laddering and TUNEL assay. For the DNA laddering analysis, cells were harvested, and the DNA was extracted and run on a gel electrophoresis as previously reported [28] TUNEL assay was performed as described [29]. The results were analyzed under a light microscope and documented on film.

RESULTS

Cloning and integration of TIMP-1 expression vectors in RMC

The PCR-generated DNA encoding TIMP-1 was cloned into pCR®2.1 vector, confirmed by DNA sequencing, and subcloned into pcDNA3.1 vector. We constructed two recombinant expression vectors: TIMP-1S, encoding the TIMP-1 sense, and TIMP-1AS, encoding the TIMP-1 antisense (Fig. 1A). TIMP-1 orientation in pcDNA3.1 was confirmed with EcoRI and Aval digestion (Fig. 1B).

To examine whether the exogenous genes have been integrated into RMC, the genomic DNA was extracted from the following four groups of cells, that is, Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, or parental RMC, respectively. PCR was used to amplify the DNA of neo^r and human TIMP-1. Our data showed that the exogenous neo^r in pcDNA3 vectors could be amplified from Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, but not parental RMC (Fig. 1C), while human TIMP-1 sense or antisense could only be amplified from TIMP-1S/MC or TIMP-1AS/MC, respectively (Fig. 1D).

RT-PCR, Northern blot and Western blot analyses of overexpressed TIMP-1S and TIMP-1AS in RMCs

Using RT-PCR, the TIMP-1S-transfected cells were expressed human TIMP-1 sense transcripts while the TIMP-1AS-transfected cells produced TIMP-1 antisense RNA (Fig. 2).

Northern blot analysis (Fig. 3) showed that the TIMP-1S-transfected cells produced a high level of TIMP-1 mRNA and Western blot analysis (Fig. 4) showed a high level of TIMP-1 protein expression. TIMP-1AS-transfected cells expressed antisense TIMP-1 mRNA and no protein expression.

Effect of over-expressed human TIMP-1S and TIMP-1AS on endogenous rat TIMP-1, MMP-2, and MMP-9

To investigate whether the transfected sense and antisense human TIMP-1 could regulate the endogenous rat

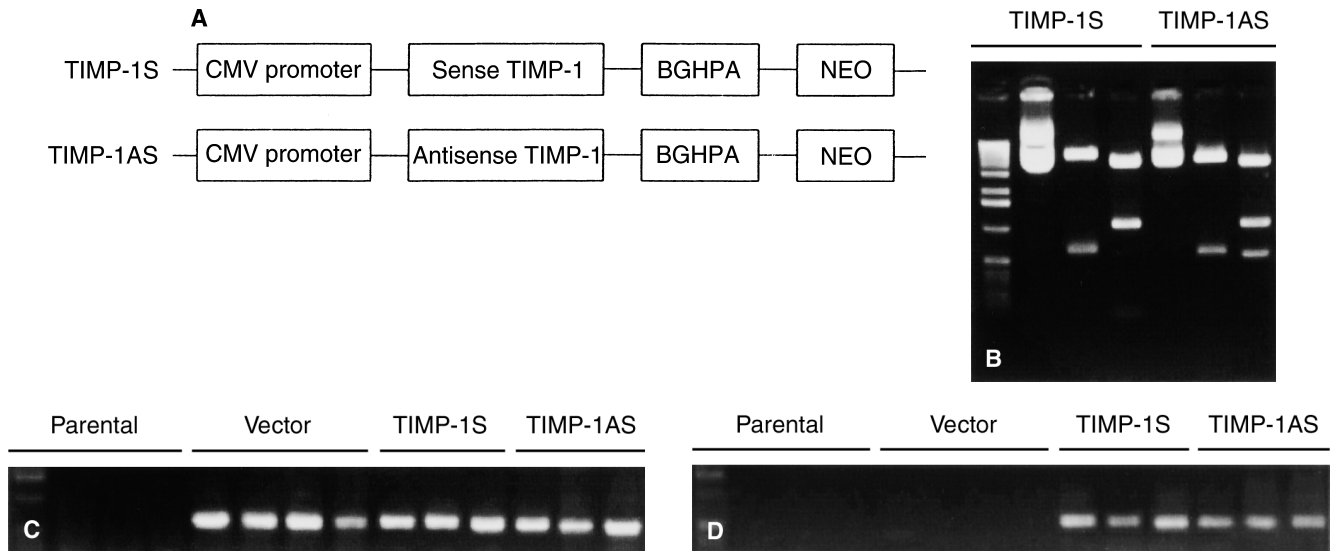


Fig. 1. Construction of the sense and antisense tissue inhibitor of metalloproteinase-1 (TIMP-1) recombinant expression vectors and their DNA integration into rat mesangial cells (RMC). (A) Scheme of sense TIMP-1 (TIMP-1S) and antisense TIMP-1 (TIMP-1AS) constructing that was used to transfect to RMC. (B) Restriction map of recombinant plasmids; TIMP-1S and TIMP-1AS recombinant plasmids were digested with EcoRI and AvaI. (C) Integration of neo^r in clones of vector/MC, TIMP-1S/MC and TIMP-1AS/MC, while there is no integration of neo^r in parental cells, as examined by PCR. (D) Integration of human TIMP-1 in clones of TIMP-1S/MC and TIMP-1 AS/MC, while there is no integration into parental cells and vector/MC, as examined by PCR.

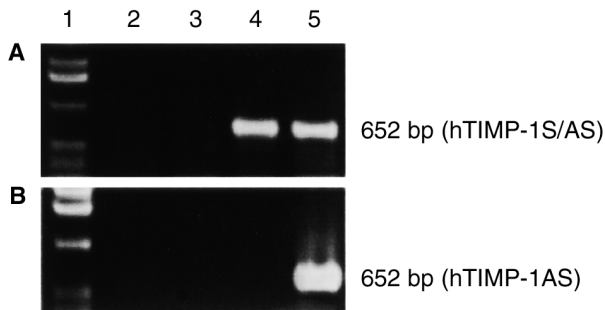


Fig. 2. Expression of exogenous human TIMP-1 sense and antisense RNA detected by RT-PCR. Cellular RNA were extracted from the following four group of cells: control parental cells (lane 2), vector/MC (lane 3), TIMP-1S/MC (lane 4), and TIMP-1AS/MC (lane 5), reverse-transcribed using an oligo (dT) primer (A), or downstream TIMP-1 primer (B). TIMP-1S/MC express sense human TIMP-1, while TIMP-1 AS/MC specifically express antisense TIMP-1. Lane 1 was the size marker.

TIMP-1, MMP-2, and MMP-9 levels in RMC, 30 µg total RNA was analyzed by Northern blot hybridization. Rat RMC normally produced weak but detectable levels of rat TIMP-1 mRNA and protein. Human sense TIMP-1 did not influence the expression of endogenous rat TIMP-1, MMP-2 and MMP-9 mRNA. However, human antisense TIMP-1 significantly reduced the endogenous rat TIMP-1 mRNA and protein, with no affection on MMP-2 and MMP-9 mRNA expression (Fig. 5).

Human TIMP-1 might protect RMC from apoptosis

Apoptosis was induced with serum deprivation Vector/MC, TIMP-1S/MC, TIMP-1AS/MC, or parental RMC.

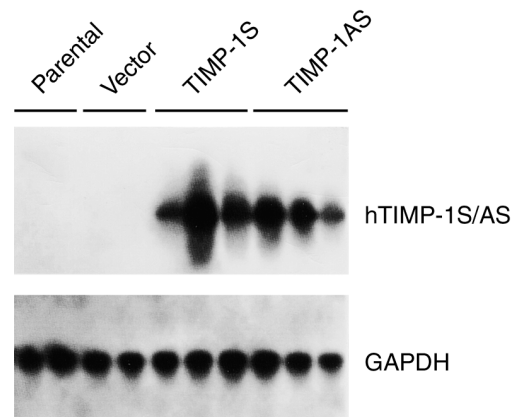


Fig. 3. Expression of sense TIMP-1 and antisense TIMP-1 detected by Northern blotting. Ten micrograms of cellular total RNA was extracted from clones of the following four group cells: control parental cells, vector/MC, TIMP-1S/MC, TIMP-1AS/MC. TIMP-1S/MC expressed sense human TIMP-1 mRNA. TIMP-1AS/MC expressed antisense TIMP-1 mRNA. No TIMP-1 expression in parental cells and vector/MC was detected. GAPDH served as an internal control for RNA expression.

Cells were observed periodically 12 to 96 hours after serum withdrawal. Apoptosis was assessed using DNA degradation methods: TUNEL assay and DNA laddering. The TIMP-1AS/MC underwent apoptosis 12 hours after serum deprivation, in contrast to parental RMC or Vector/MC, which only underwent apoptosis 48 hours after serum deprivation. TIMP-1S/MC did not undergo apoptosis until four days later (Fig. 6).

Using DNA laddering, we analyzed the DNA frag-

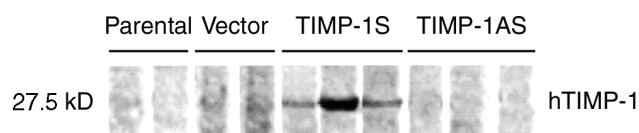


Fig. 4. Expression of TIMP-1 protein detected by Western blotting. Ten micrograms of protein from cells of the same four groups as in Figure 3 were subjected to Western blot analysis using monoclonal mouse anti-human TIMP-1 antibody. Numbers at the left correspond to relative molecular weight of protein in kilodalton. Cells transfected with sense TIMP-1 expressed the TIMP-1 protein. All the cell clones for each group had similar results.

mentation of cells. TIMP-1AS/MC showed typical DNA ladder 24 hours after serum starvation, parental cells and Vector/MC showed DNA ladder 60 hours after serum withdrawal, while TIMP-1S/MC still showed genomic DNA (Fig. 7).

Effect of TIMP-1 antibody and BB-94 on apoptosis

To investigate the specificity of TIMP-1 anti-apoptotic effect, we incubated parental RMC, vector/MC, TIMP-1S/MC, and TIMP-1AS/MC with synthetic MMP inhibitor BB-94 and with anti-TIMP-1 monoclonal antibody. Neutralization of secreted TIMP-1 with TIMP-1 antibody restored the sensitivity of TIMP-1S/MC to apoptosis induced by serum deprivation (Fig. 8). Forty-eight hours after cultured in serum-free medium, the percentage of apoptotic cells was increased dramatically, from less than 5% to 67%. Parental, vector/MC and TIMP-1AS/MC all had a slight increase in apoptotic cells at 24 hours after serum deprivation.

In contrast, when parental RMC, Vector/MC, TIMP-1S/MC, or TIMP-1AS/MC was treated with synthetic MMP inhibitor BB-94, no significant changes were observed in apoptosis of these cells (data not shown), demonstrating that inhibition of MMP function did not affect the effects of TIMP-1 on apoptosis in RMC induced by serum deprivation. These results suggested that the anti-apoptotic effect of TIMP-1 might be independent on its inhibition on MMP function.

Effects of sense and antisense TIMP-1 on the expressions of apoptosis-related proteins

Bcl-2 and Bax have been shown to be involved in the apoptosis of many kinds of cells [30]. Bcl-2 has been shown to act as an inhibitor of apoptosis [31], while Bax acts in opposition to Bcl-2 and overexpression of Bax allows apoptosis to proceed [32]. To determine whether Bcl-2 or Bax are implicated in the cytoprotective effect of TIMP-1, Northern hybridization was performed using these four groups of RMC to detect the expression of Bcl-2 and Bax mRNA. Although all these cells expressed Bcl-2, up-regulation and down-regulation of TIMP-1 had no effect on the basal level of Bcl-2 expression. In contrast, up-regulation of TIMP-1 decreased the expression of Bax

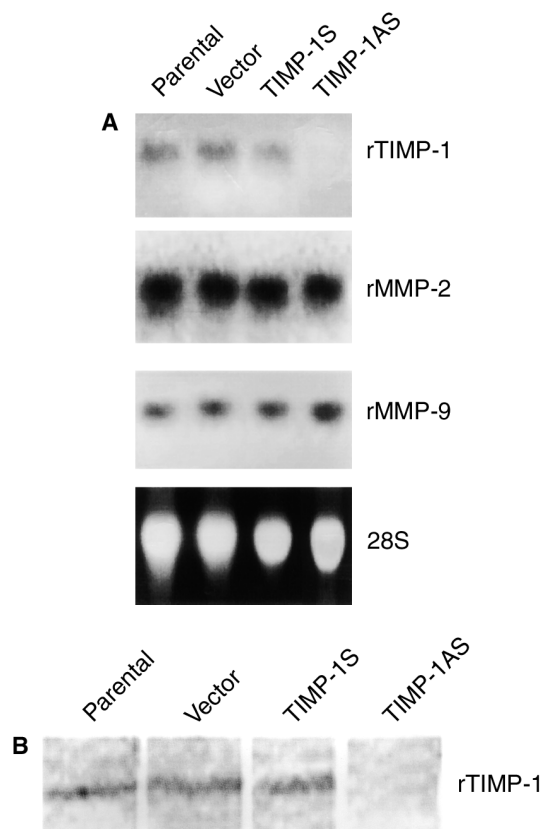


Fig. 5. Effects of human sense and antisense TIMP-1 on the endogenous rat TIMP-1, MMP-2, MMP-9 expression. (A) Thirty micrograms of RNA extracted from control parental cells, vector/MC, TIMP-1S/MC, TIMP-1AS/MC were detected by Northern blot. Neither sense nor antisense human TIMP-1 transfection had any effects on the endogenous rat MMP-2 and MMP-9 mRNA expression. Normally, there was weak expression of endogenous TIMP-1 expression in RMC, transfection with sense human TIMP-1 had no effect on the endogenous TIMP-1 expression, while antisense human TIMP-1 transfection reduced the endogenous rat TIMP-1 expression. (B) Fifty microliters of protein from control parental cells, vector/MC, TIMP-1S/MC, and TIMP-1AS/MC was subjected to Western blot analysis. Monoclonal goat anti-rat TIMP-1 was used for Western blot, the rat endogenous TIMP-1 was reduced by antisense TIMP-1 transfection. Data are representative of experiments with all of the cell clones from each group.

and down-regulation of TIMP-1 enhanced the expression of Bax. These results suggested that Bax might, at least in part, be involved in the effect of TIMP-1 on apoptosis in RMC induced by serum deprivation (Fig. 9).

DISCUSSION

The present study did not investigate the effects of TIMP-1 on metabolism of extracellular matrix, but found that a high level of TIMP-1 expression was able to effectively inhibit apoptosis of RMC in vitro, suggesting that TIMP-1 might have a novel function of suppressing apoptosis of RMC.

Until now, it has been found that glomerular mesangial cells, glomerular epithelial cells, proximal tubular epithe-

lial cells and fibroblasts in renal interstitium can express TIMP-1 [33, 34]. These data demonstrated that TIMP-1 is widely expressed in the kidney. TIMP-1 is a very important factor in renal diseases due to its role in MMP inhibition. Many studies have reported that the expression of TIMP-1 is significantly increased in various rodent renal fibrosis models [35–38], and clinical studies also have shown that the expression of TIMP-1 in kidney was up-regulated in patients with glomerulosclerosis [39]. Extracellular matrix accumulation and the apoptosis of mesangial cell are two key events during the process of glomerular diseases. The effects of TIMP-1 on ECM metabolism in kidney has been well investigated, and it is clear now that TIMP-1 plays an important role in the development of renal diseases by its ability to inhibit MMPs' functions [1–4]. However, these studies of TIMP-1 in kidney had focused on its role in inhibiting the ECM-degrading activities of MMPs, and it is still unknown whether TIMP-1 has other functions beyond blocking MMPs' activities in the kidney.

Studies have shown that TIMP-1 expression is associated with resistance to apoptosis of Burkitt's lymphoma cell lines and human breast epithelial cells [40, 41]. The effects on apoptosis of other TIMPs also have been described. TIMP-2 inhibits human melanoma cell invasion, and TIMP-2 over-expression in tumor clones was found to be more resistant to apoptosis than parental and control melanoma cells [42]. Another report demonstrated that TIMP-3 could promote apoptosis in rat vascular smooth muscle cells [43]. All these studies support the idea that TIMPs can exert different functions, such as modulation of apoptosis and inhibition of MMPs' activities. Apoptosis of glomerular mesangial cells is important in the process of glomerulosclerosis. Although studies have shown that TIMP-1 can inhibit apoptosis of B cells [15], it remains unknown whether TIMP-1 has any effect on apoptosis of renal cells. Since glomerular mesangial cells play a key role in various renal diseases [2], it is worthwhile to address the effect of TIMP-1 on apoptosis of mesangial cells. In present study, we found that high level of intrinsic TIMP-1 expression effectively inhibited apoptosis in RMC in vitro, suggesting that one function of TIMP-1 may be to suppress the apoptosis of RMC.

Antisense oligos is believed to bind the endogenous transcript with an ultimate down-regulation of the particular protein level [44], and the role of antisense TIMP-1 in tumorigenics and metastasis has been reported [45, 46]. In the studies of gene transfection and transgenic mice, it is a common and well-accepted method to transfect human genes into animal cells in vivo or in vitro. To provide data for the exploration of role of TIMP-1 in human renal diseases, we cloned human *TIMP-1*, constructed two contrasting recombinant expression vectors, TIMP-1S and TIMP-1AS, and transfected them into RMC, the results demonstrated that both sense and antisense human

TIMP-1 could stably integrated into RMC. TIMP-1S/MC could effectively express human TIMP-1 mRNA and protein but have no effect on the endogenous rat TIMP-1 expression, while TIMP-1AS/MC could express antisense TIMP-1 and significantly down-regulate endogenous rat TIMP-1 mRNA and protein expression. Hence, we established two contrasting RMC models with constitutive over-expression or reduction of TIMP-1. In most prior studies, up-regulation or down-regulation of TIMP-1 in cultured cells was by the addition of exogenous recombinant TIMP-1 or specific TIMP-1 antibodies into culture media. Our studies, however, focused on antisense technology-based genetic modulation of cells of kidney, which provided effective models for studying apoptosis of RMC.

Our data demonstrated that the effect of inhibiting apoptosis of RMC might be a novel function of TIMP-1 in RMC, and that this effect was TIMP-1-specific and not secondary to MMPs' inhibition. Firstly, neither sense nor antisense TIMP-1 transfection affected the expression of rat MMP-2 or rat MMP-9, but both substantially affected the apoptosis of RMC. Secondly, while synthetic inhibitor BB-94, which can completely inhibit a wide spectrum of MMPs, had no effects on apoptosis of RMC, neutralization of TIMP-1 with specific TIMP-1 antibody apparently affect apoptosis of RMC. Reduced-alkylated TIMP-1, completely devoid of MMP inhibitory activity, still resulted in apoptosis suppression [46]. The result that the anti-apoptotic effect of TIMP-1 was independent of its MMP inhibitory effect, which was inconsistent with results of other studies, demonstrated that the cell growth modulatory effect of TIMP-1 was unrelated to its MMPs' inhibiting ability [47]. The above data suggest that in addition to inhibiting MMPs' activities, TIMP-1 also may be able to regulate apoptosis of cells through another pathway. Our data combining the results of other reports suggest that TIMP-1 has the ability to directly regulate apoptosis.

TUNEL and DNA laddering are two classical and specific assays in detecting DNA fragmentation of apoptosis [48]. Since the aim of our study was to observe if TIMP-1 could inhibit apoptosis of RMC, we examined the alterations of apoptotic cells' morphology as well as the specific DNA fragmentations with TUNEL and DNA laddering assays that showed enough data of apoptosis to support our conclusion.

In the present study, apoptosis was investigated in parental RMC, Vector/MC, TIMP-1S/MC, and TIMP-1AS/MC. It was shown that some TIMP-1AS/MC underwent apoptosis after 12 hours culture in serum free media and almost all of the TIMP-1AS/MC underwent apoptosis after 24 hours. However, TIMP-1S/MC did not undergo apoptosis until four days after serum deprivation. The results disclosed that TIMP-1S was active in blocking apoptosis while TIMP-1AS promoted apoptosis

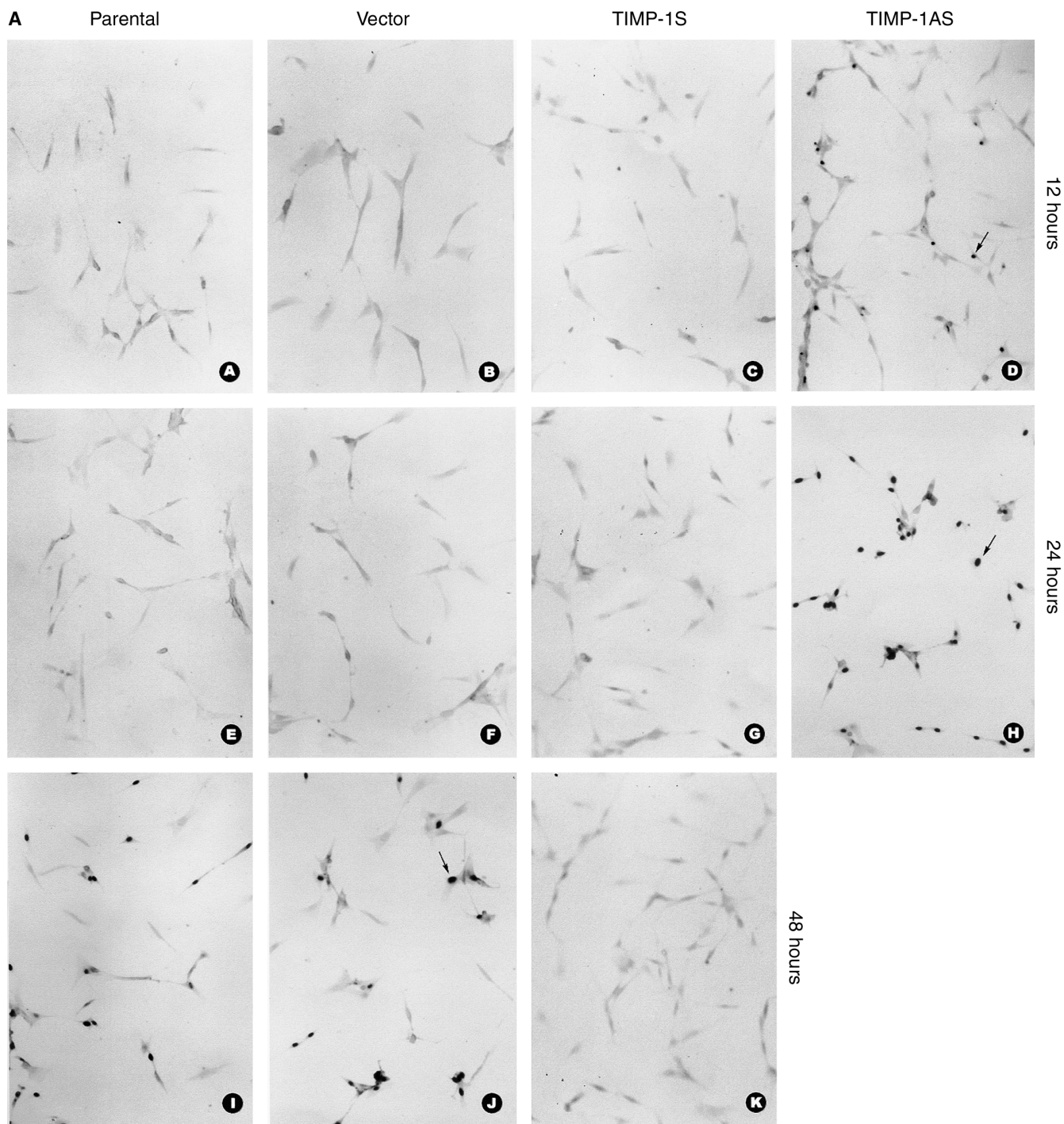


Fig. 6. (A) Effects of sense and antisense TIMP-1 on apoptosis in RMC induced by serum deprivation: TUNEL staining detecting the apoptosis. (A, B, C, D) Twelve hours after culture in serum-free medium. (E, F, G, H) Twenty-four hours after culture in serum-free medium. (I, J, K) Forty-eight hours after culture in serum-free medium. (A, E, I) Parental cells underwent apoptosis after 48 hours of serum deprivation. (B, F, J) Vector/MC also underwent apoptosis after 48 hours of serum deprivation. (C, G, K) TIMP-1S/MC did not undergo apoptosis after 48 hours of serum deprivation. (D, H) TIMP-1AS/MC began to undergo apoptosis after 12 hours of serum deprivation. The arrows indicated positive cells of TUNEL staining.

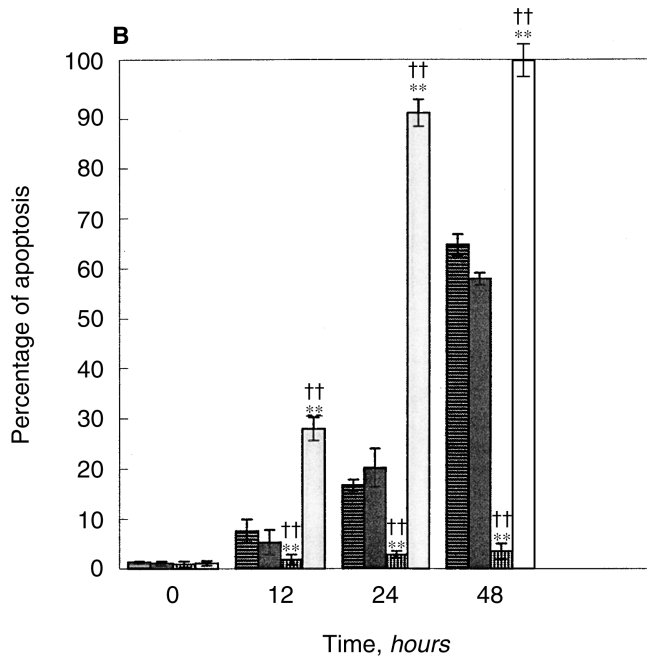


Fig. 6. (Continued). (B) Effects of sense and antisense TIMP-1 on apoptosis in RMC induced by serum deprivation: Percentage of apoptotic as examined by TUNEL method. Symbols are: (■) parental cells; (■) vector/MC; (■) TIMP-1S/MC; (□) TIMP-1AS/MC. ** $P < 0.001$ compared with the parental cell group and $\dagger\dagger P < 0.001$ vector/MC group. $N = 8$ in each group.

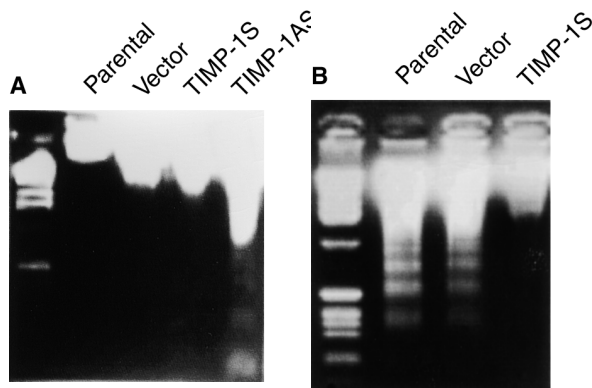


Fig. 7. DNA laddering for apoptosis detection. Extracted genomic DNA from parental, vector, TIMP-1S, and TIMP-1AS-transfected RMC 24 hours after serum deprivation. TIMP-1AS/MC showed typical DNA ladder (A) and 60 hours later, parent cells and vector/MC showed DNA ladder, while in the meantime the TIMP-1S/MC still remained genomic DNA (B).

of RMC. As over-proliferation of RMC is a very important event in many glomerular diseases, the fact that TIMP-1AS promoted apoptosis of RMC not only suggests a novel function TIMP-1, but also encourages design of experiments use TIMP-1AS to treat some glomerular mesangioproliferative diseases.

Although several reports have shown that TIMP-1 inhibited apoptosis of in some types of cells, little is known

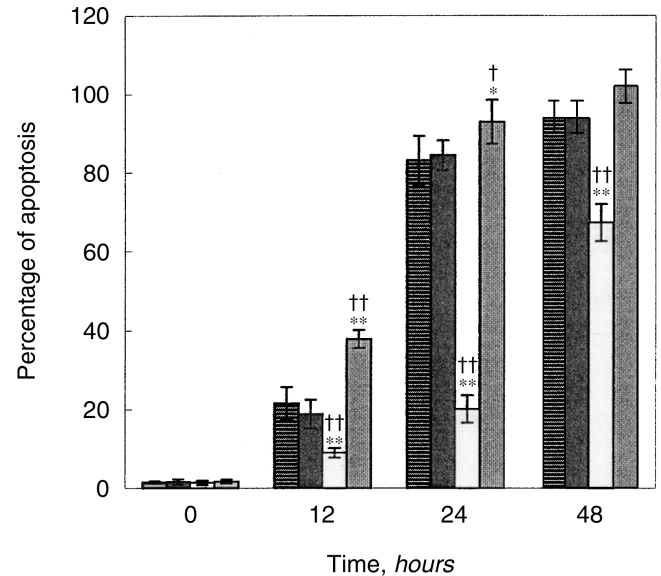


Fig. 8. Effect of TIMP-1 antibody on apoptosis in RMC induced by serum deprivation ($N = 8$). All the cells were treated with TIMP-1 neutralizing antibody before the induction of apoptosis by serum deprivation. * $P < 0.05$ compared with the parental cell group and vector/MC group. ** $P < 0.001$ compared with the parental cell group and vector/MC group. Symbols are: (■) parental cells; (■) vector/MC; (□) TIMP-1S/MC; (■) TIMP-1AS/MC.

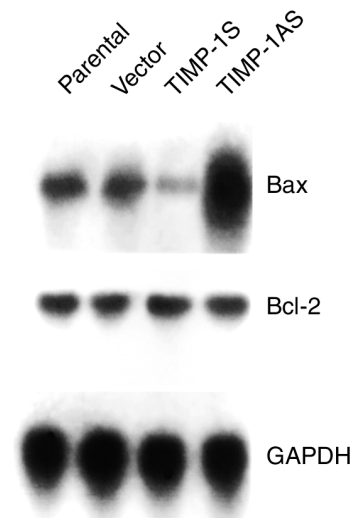


Fig. 9. Effects of sense and antisense TIMP-1 transfection on expression of apoptotic protein. Twenty micrograms of cellular total RNA was used for Northern blot analyses. Sense or antisense TIMP-1 transfection had no effect on the expression of Bcl-2. However, up-regulation of TIMP-1 decreased the expression of Bax, and down-regulation of TIMP-1 enhanced Bax expression.

about the mechanism. It has been reported that TIMP-1 inhibited the apoptosis induced by cold shock, serum deprivation and γ -radiation in B cells, and suppressed the apoptosis caused by hydrogen peroxide, adriamycin and x-ray irradiation in human breast epithelial cells [49].

The phenomenon that TIMP-1 protects against apoptosis induced by various stresses and chemicals suggests that TIMP-1 may interfere with a common signaling pathway of the apoptotic cascade.

We treated TIMP-1S/MC with synthetic MMP inhibitor BB-94 and anti-TIMP-1 monoclonal antibody to investigate whether the anti-apoptotic ability of TIMP-1 is dependent on its MMP inhibition function. Our results demonstrated that neutralization of secreted TIMP-1 with TIMP-1 antibody could restore the RMC sensitivity to apoptosis, while BB-94 had no effect on the apoptosis, suggesting that the anti-apoptotic effect of TIMP-1 might be independent of its MMP inhibition function [50].

The Bcl-2 family of proto-oncogenes encodes specific proteins that regulate programmed cell death on different physiological and pathological conditions [30]. Among them, Bcl-2 suppresses apoptosis [31]. Bax, another member of the Bcl-2 family, which is widely expressed in vivo and is subject to tissue-specific differentiation of stage-dependent regulation, accelerates apoptotic cell death [32]. Thus, we further detected the effect of TIMP-1 on the expression of apoptosis-related genes *Bcl-2* and *Bax*. Although up-regulation of TIMP-1 had no effect on Bcl-2 expression, it decreased Bax expression indeed. This result suggests that the modulation of TIMP-1 on apoptosis of RMC might have a correlation with the gene *Bax*.

In summary, we established two stably transfected RMCs, which had an over-expression or reduction of human TIMP-1 expression. The data demonstrate that apoptosis of RMC induced by serum deprivation could be suppressed by TIMP-1S transfection, which might be related to the apoptosis-related gene *Bax*.

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